



pcDNA[™] Gateway[®] Directional TOPO[®] Expression Kits

**Five-minute, directional TOPO[®] Cloning of blunt-end
PCR products into mammalian expression vectors
for the Gateway[®] Technology**

Catalog nos. K2440-20 and K2460-20

Version E
31 October 2010
25-0441

**A Limited Use Label License covers this product (see Purchaser Notification).
By use of this product, you accept the terms and conditions of the Limited
Use Label License.**

User Manual

Table of Contents

Table of Contents	iii
TOPO® Cloning Procedure for Experienced Users	v
Kit Contents and Storage	vi
Accessory Products.....	ix
Introduction	1
Overview.....	1
How Directional TOPO® Cloning Works	3
Experimental Outline	4
Methods	5
Designing PCR Primers	5
Producing Blunt-End PCR Products.....	9
Performing the TOPO® Cloning Reaction	10
Transforming One Shot® TOP10 Competent Cells.....	12
Analyzing Transformants.....	15
Transfecting Cells	17
Detecting Recombinant Fusion Proteins	18
Creating Stable Cell Lines.....	20
Generating an Entry Clone	22
Troubleshooting.....	23
Appendix.....	25
Performing the Control Reactions	25
Gel Purifying PCR Products.....	27
Recipes.....	29
Blasticidin.....	31
Map and Features of pcDNA/V5/GW/D-TOPO® Vectors	32
Map of pcDNA™3.2/V5/GW-CAT and pcDNA™6.2/V5/GW-CAT	34
Technical Service.....	35
Purchaser Notification	36
Gateway® Clone Distribution Policy	39
Product Qualification	40
References	41

TOPO[®] Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the directional TOPO[®] Cloning procedure. If you are performing the TOPO[®] Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

Step	Action										
Design PCR Primers	<ul style="list-style-type: none"> • Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5' end of the forward primer. • Design the primers such that your gene of interest will be optimally expressed and fused in frame with any epitope tags, if desired. 										
Amplify Your Gene of Interest	<ol style="list-style-type: none"> 1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product. 2. Use agarose gel electrophoresis to check the integrity of your PCR product. 										
Perform the TOPO [®] Cloning Reaction	<ol style="list-style-type: none"> 1. Set up the following TOPO[®] Cloning reaction. For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO[®] vector. Note: If you plan to transform electrocompetent <i>E. coli</i>, use Dilute Salt Solution in the TOPO[®] Cloning reaction. <table style="margin-left: 20px; border: none;"> <tr> <td>Fresh PCR product</td> <td style="text-align: right;">0.5 to 4 μl</td> </tr> <tr> <td>Salt Solution</td> <td style="text-align: right;">1 μl</td> </tr> <tr> <td>Water</td> <td style="text-align: right;">add to a final volume of 5 μl</td> </tr> <tr> <td><u>TOPO[®] vector</u></td> <td style="text-align: right;"><u>1 μl</u></td> </tr> <tr> <td>Total volume</td> <td style="text-align: right;">6 μl</td> </tr> </table> 2. Mix gently and incubate for 5 minutes at room temperature. 3. Place on ice and proceed to transform One Shot[®] TOP10 chemically competent <i>E. coli</i>, below. 	Fresh PCR product	0.5 to 4 μ l	Salt Solution	1 μ l	Water	add to a final volume of 5 μ l	<u>TOPO[®] vector</u>	<u>1 μl</u>	Total volume	6 μ l
Fresh PCR product	0.5 to 4 μ l										
Salt Solution	1 μ l										
Water	add to a final volume of 5 μ l										
<u>TOPO[®] vector</u>	<u>1 μl</u>										
Total volume	6 μ l										
Transform TOP10 Chemically Competent <i>E. coli</i>	<ol style="list-style-type: none"> 1. Add 2 μl of the TOPO[®] Cloning reaction into a vial of One Shot[®] TOP10 chemically competent <i>E. coli</i> and mix gently. 2. Incubate on ice for 5 to 30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 μl of room temperature S.O.C. medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 50-200 μl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C. 										

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kits to perform the control reaction. See the protocol on pages 25-26 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Quantity	Catalog no.
pcDNA™3.2/V5 Gateway® Directional TOPO® Expression Kit	20 reactions	K2440-20
pcDNA™6.2/V5 Gateway® Directional TOPO® Expression Kit	20 reactions	K2460-20



Important

The pcDNA3.2/V5/GW/D-TOPO® and pcDNA6.2/V5/GW/D-TOPO® vectors have been renamed to be more descriptive and to better reflect the functionality of the vector.

Shipping/Storage

The pcDNA™ Gateway® Directional TOPO® Expression Kits are shipped on dry ice. Each kit contains two boxes as described below. Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pcDNA™ Gateway® Directional TOPO® Reagents	-20°C or -80°C
2	One Shot® TOP10 Chemically Competent <i>E. coli</i>	-80°C

continued on next page

Kit Contents and Storage, continued

pcDNA™ Gateway® Directional TOPO® Reagents

pcDNA™ Gateway® Directional TOPO® reagents (Box 1) are listed below. Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.

Store Box 1 at -20°C or -80°C.

Item	Concentration	Amount
Gateway® TOPO® Vector (pcDNA3.2/V5/GW/D-TOPO® or pcDNA6.2/V5/GW/D-TOPO®)	15-20 ng/μl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 μg/ml BSA 30 μM bromophenol blue	20 μl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 μl
Salt Solution	1.2 M NaCl 0.06 M MgCl ₂	50 μl
Water	---	1 ml
T7 Promoter Primer	0.1 μg/μl in TE Buffer, pH 8	20 μl
TK polyA Reverse Primer	0.1 μg/μl in TE Buffer, pH 8	20 μl
Control PCR Primers	0.1 μg/μl each in TE Buffer, pH 8	10 μl
Control PCR Template	0.1 μg/μl in TE Buffer, pH 8	10 μl
Control Plasmid (pcDNA™3.2/V5/GW-CAT or pcDNA™6.2/V5/GW-CAT)	0.5 μg/μl in TE, pH 8.0	10 μl

Sequences of the Primers

The table below provides the sequences of the T7 Promoter and TK polyA Reverse sequencing primers.

Primer	Sequence	pMoles Supplied
T7 Promoter Primer	5'-TAATACGACTCACTATAGGG-3'	328
TK polyA Reverse Primer	5'-CTTCCGTGTTTCAGTTAGC-3'	348

continued on next page

Kit Contents and Storage, continued

One Shot® TOP10 Chemically Competent Cell Reagents

The table below lists the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is 1×10^9 cfu/ μ g DNA. Store Box 2 at -80°C .

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature or $+4^\circ\text{C}$)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl_2 10 mM MgSO_4 20 mM glucose	6 ml
TOP10 cells	--	21 x 50 μ l
pUC19 Control DNA	10 pg/ μ l in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 μ l

Genotype of TOP10

F mcrA $\Delta(mrr\text{-}hsdRMS\text{-}mcrBC)$ $\Phi 80lacZ\Delta M15$ $\Delta lacX74$ *recA1* *araD139* $\Delta(ara\text{-}leu)7697$ *galU galK rpsL* (Str^R) *endA1 nupG*

Accessory Products

Introduction

The products listed in this section may be used with the pcDNA™ Gateway® Directional TOPO® Expression Kits. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 35).

Additional Products

Many of the reagents supplied in the pcDNA™ Gateway® Directional TOPO® Expression Kits and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
One Shot® TOP10 Chemically Competent <i>E. coli</i>	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent <i>E. coli</i>	10 reactions	C4040-50
Lipofectamine™ 2000	0.75 ml	11668-027
	1.5 ml	11668-019
Ampicillin	20 ml	11593-019
Geneticin®	1 g	11811-023
	5 g	11811-031
	20 ml (50 mg/ml)	10131-035
	100 ml (50 mg/ml)	10131-027
Blasticidin	50 mg	R210-01
S.O.C. Medium	10 x 10 ml	15544-034
CAT Antiserum*	50 µl	R902-25
Phosphate Buffered Saline, pH 7.4	500 ml	10010-023
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Gateway® BP Clonase™ Enzyme Mix	20 reactions	11789-013
	100 reactions	11789-021
pDONR™201	6 µg	11798-014
pDONR™221	6 µg	12536-017
pDONR™/Zeo	6 µg	12535-035
PCR Cloning System with Gateway® Technology with pDONR™221	1 kit	12535-019
PCR Cloning System with Gateway® Technology with pDONR™/Zeo	1 kit	12535-027

*The amount supplied is sufficient to perform 25 Western blots using 10 ml working solution per reaction.

continued on next page

Accessory Products, continued

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using Anti-V5 antibodies available from Invitrogen. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods. The fluorescein isothiocyanate (FITC)-conjugated antibody allows one-step detection in immunofluorescence experiments.

The amount of antibody supplied is sufficient for 25 Western blots or 25 immunostaining reactions (FITC-conjugated antibody only).

Product	Epitope	Catalog no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern <i>et al.</i> , 1991).	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-V5-FITC Antibody		R963-25

Introduction

Overview

Introduction

The pcDNA™ Gateway® Directional TOPO® Expression Kits utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector which may be used with the Gateway® Technology available from Invitrogen. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

Features of the pcDNA™ Gateway® TOPO® Vectors

The pcDNA3.2/V5/GW/D-TOPO® and pcDNA6.2/V5/GW/D-TOPO® vectors are designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for high-level expression in mammalian cells and for entry into the Gateway® Technology. Features of the vectors include:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells
 - *attB1* and *attB2* sites for site-specific recombination of the expression clone with a Gateway® donor vector to generate an entry clone
 - Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
 - The V5 epitope tag for detection using Anti-V5 antibodies
 - The Herpes Simplex Virus thymidine kinase polyadenylation signal for proper termination and processing of the recombinant transcript
 - *f1* intergenic region for production of single-strand DNA in F plasmid-containing *E. coli*
 - SV40 early promoter and origin for expression of the neomycin (pcDNA3.2/V5/GW/D-TOPO®) or Blasticidin (pcDNA6.2/V5/GW/D-TOPO®) resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
 - Neomycin or Blasticidin resistance gene (pcDNA3.2/V5/GW/D-TOPO® and pcDNA6.2/V5/GW/D-TOPO®, respectively) for selection of stable cell lines
 - The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
 - The ampicillin (*bla*) resistance gene for selection in *E. coli*
-

continued on next page

Overview, continued

The Gateway[®] Technology

The Gateway[®] Technology is a universal cloning system that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest, simply TOPO[®] Clone your blunt-end PCR product into a pcDNA[™] Gateway[®] Directional TOPO[®] vector and transfect your expression clone into the mammalian cell line of choice.

To express your gene of interest in any other expression system:

1. Generate an entry clone by performing a BP recombination reaction between your expression clone and a Gateway[®] donor vector.
2. Perform an LR recombination reaction between the entry clone and a variety of Gateway[®] destination vectors to generate an expression construct to express your protein of interest in virtually any expression system.

For more information about the Gateway[®] Technology, refer to the Gateway[®] Technology manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).

Tag-On-Demand[™] System

The pcDNA[™] Gateway[®] Directional TOPO[®] vectors are compatible with the Tag-On-Demand[™] System which allows expression of both native and C-terminally-tagged recombinant protein from the same expression construct.

The System is based on stop suppression technology originally developed by RajBhandary and colleagues (Capone *et al.*, 1985) and consists of a recombinant adenovirus expressing a tRNA^{ser} suppressor. When an expression vector encoding a gene of interest with the TAG (amber stop) codon is transfected into mammalian cells and the tRNA^{ser} suppressor supernatant is present, the stop codon will be translated as serine, allowing translation to continue and resulting in production of a C-terminally-tagged fusion protein.

For more information, refer to the Tag-On-Demand[™] Suppressor Supernatant manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).

How Directional TOPO[®] Cloning Works

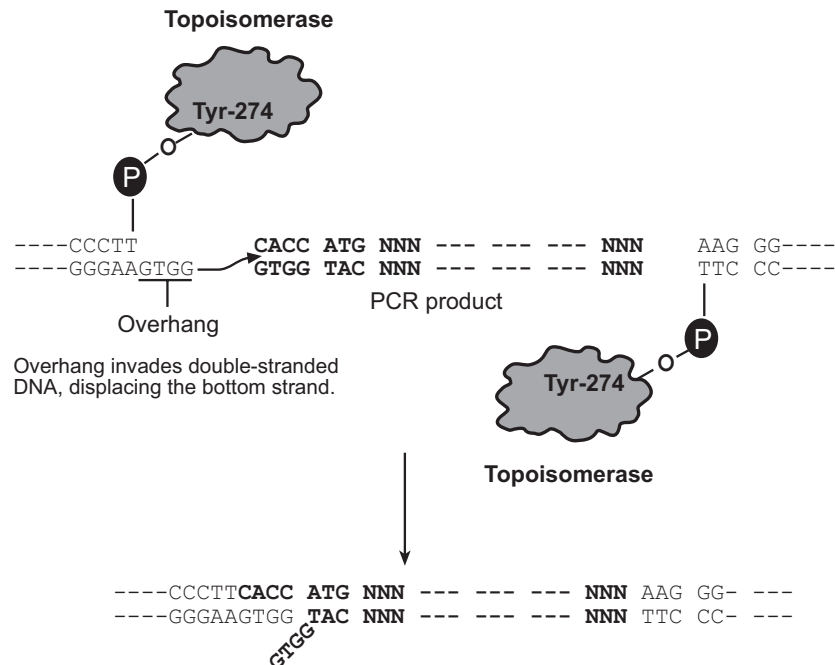
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO[®] Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO[®] Cloning

Directional joining of double-strand DNA using TOPO[®]-charged oligonucleotides occurs by adding a 3' single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5' end of the TOPO[®]-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO[®]-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5' end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



Experimental Outline

Experimental Outline

The table below describes the general steps needed to clone and express your gene of interest. For more details, refer to the pages indicated.

Step	Action	Page
1	Design PCR primers to clone your gene of interest in frame with the C-terminal V5 epitope tag (if desired). Consult the diagram on page 8 to help you design your PCR primers.	5-8
2	Produce your blunt-end PCR product.	9
3	TOPO [®] Clone your PCR product into pcDNA3.2/V5/GW/D-TOPO [®] or pcDNA6.2/V5/GW/D-TOPO [®] and transform into One Shot [®] TOP10 <i>E. coli</i> . Select transformants on LB plates containing 50-100 µg/ml ampicillin.	10-14
4	Analyze transformants by restriction digestion or PCR.	15
5	Select a transformant with the correct restriction pattern and sequence it to confirm that your gene is cloned in frame with the C-terminal V5 epitope tag (if desired).	15
6	Transfect your pcDNA3.2/V5/GW/D-TOPO [®] or pcDNA6.2/V5/GW/D-TOPO [®] construct into a mammalian host cell line using your method of choice.	17
7	Assay for expression of your protein of interest.	18-19

Methods

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
 - Sequences required for proper translation initiation of your PCR product
 - Whether or not you wish your PCR product to be fused in frame with the C-terminal V5 epitope tag
-

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to page 8 for a diagram of the TOPO[®] Cloning site for the pcDNA[™] Gateway[®] Directional TOPO[®] vectors.

- To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5' end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pcDNA/V5/GW/D-TOPO[®] vector.
- Make sure your sequence of interest includes a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is **(G/A)NNATGG**. Other sequences are possible, but the G or A at position -3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5' end of the primer (see **Example** below).

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5'-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5'-C **ACC** ATG **GGA** TCT GAT AAA

If you design the forward PCR primer as noted above, then the ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.



Note

The first three base pairs of the PCR product following the 5' CACC overhang will constitute a functional codon.

continued on next page

Designing PCR Primers, continued

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the following points below. Refer to page 8 for a diagram of the TOPO[®] Cloning site for the pcDNA[™] Gateway[®] Directional TOPO[®] vectors.

- To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer **MUST NOT** be complementary to the overhang sequence GTGG at the 5' end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 below). We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with the C-terminal V5 epitope tag, design the reverse PCR primer to remove the native stop codon in the gene of interest (see Example #2 on the next page).

Note: If you will be using your pcDNA/V5/GW/D-TOPO[®] construct with the Gateway[®] Technology, note that the gene of interest will remain in frame with any C-terminal tags provided by the destination vector following the LR recombination reaction.

- If you **do not** wish to fuse your PCR product in frame with the C-terminal V5 epitope tag, include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see Example #2 on the next page).

Note: If you will be using your pcDNA/V5/GW/D-TOPO[®] construct with the Gateway[®] Technology, note that the stop codon will be carried over into the entry clone following the BP recombination reaction.

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag. The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TGA-3'

One possibility is to design the reverse PCR primer to start with the codon just up-stream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

Another possibility is to design the reverse primer so that it hybridizes just down-stream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine (see below).

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TGA-3'

Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5'

continued on next page

Designing PCR Primers, continued

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

...GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TGA-3'

- To fuse the ORF in frame with a C-terminal tag, remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

5'-TGC AGT CGT CGA GTG CTC CGA CTT-3'

This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.

- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

5'-TCA TGC AGT CGT CGA GTG CTC CGA CTT-3'



Important

- Remember that the pcDNA/V5/GW/D-TOPO® vectors accept blunt-end PCR products.
 - Do not add 5' phosphates to your primers for PCR. This will prevent ligation into the pcDNA/V5/GW/D-TOPO® vectors.
 - We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).
-

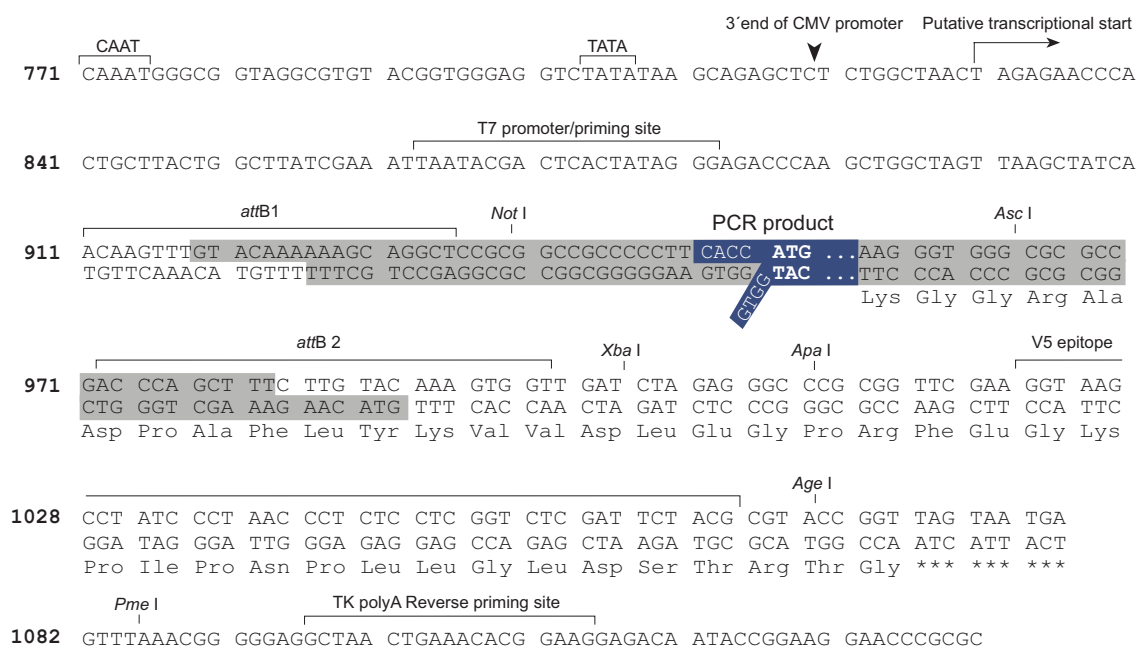
continued on next page

Designing PCR Primers, continued

TOPO® Cloning Site

Use the diagram below to help you design PCR primers to clone your PCR product into a pcDNA/V5/GW/D-TOPO® vector. Restriction sites are labeled to indicate the cleavage site. The shaded region corresponds to sequences that will be transferred from the pcDNA/V5/GW/D-TOPO® vector into the entry clone following the BP recombination reaction. **The sequences of pcDNA3.2/V5/GW/D-TOPO® and pcDNA6.2/V5/GW/D-TOPO® are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).**

Note: If you are using the pcDNA/V5/GW/D-TOPO® vector in the Tag-On-Demand™ System, your gene of interest must contain a TAG stop codon (see page 2 for more information).



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Needed

You should have the following materials on hand before beginning.

Note: dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
 - 10X PCR buffer appropriate for your polymerase
 - DNA template and primers to produce the PCR product
-

Producing Blunt-End PCR Products

Set up a 25 μ l or 50 μ l PCR reaction using the guidelines below.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
 - Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
 - Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
 - After cycling, place the tube on ice or store at -20°C for up to 2 weeks. Proceed to **Checking the PCR Product**, below.
-

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 27-28).
 - Estimate the concentration of your PCR product. You will use this information when setting up your TOPO[®] Cloning reaction (see **Amount of PCR Product to Use in the TOPO[®] Cloning Reaction**, next page for details).
-

Performing the TOPO[®] Cloning Reaction

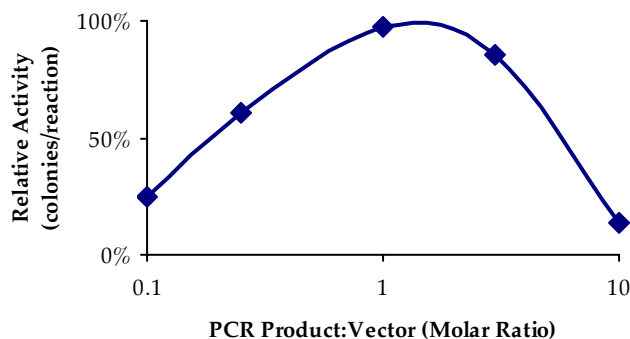
Introduction

Once you have produced the desired PCR product, you are ready to TOPO[®] Clone it into a pcDNA[™] Gateway[®] Directional TOPO[®] vector and transform the recombinant vector into TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot[®] TOP10 Competent Cells** (pages 12-14) before beginning. If this is the first time you have TOPO[®] Cloned, perform the control reactions on pages 25-26 in parallel with your samples.

Amount of PCR Product to Use in the TOPO[®] Cloning Reaction

When performing directional TOPO[®] Cloning, we have found that the molar ratio of PCR product:TOPO[®] vector used in the reaction is critical to its success. **To obtain the highest TOPO[®] Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO[®] vector (see figure below).** Note that the TOPO[®] Cloning efficiency decreases significantly if the ratio of PCR product: TOPO[®] vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO[®] Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO[®] Cloning.

Tip: For the pcDNA[™] Gateway[®] Directional TOPO[®] vectors, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO[®] Cloning reaction generally results in a suitable number of colonies.



continued on next page

Performing the TOPO[®] Cloning Reaction, continued

Using Salt Solution in the TOPO[®] Cloning Reaction

You will perform TOPO[®] Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO[®] Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page ix for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO[®] Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO[®] Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM MgCl₂ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM MgCl₂ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO[®] Cloning reaction as directed below.

Performing the TOPO[®] Cloning Reaction

Use the procedure below to perform the TOPO[®] Cloning reaction. Set up the TOPO[®] Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli*. **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO[®] vector in your TOPO[®] Cloning reaction.

Note: The blue color of the TOPO[®] vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent <i>E. coli</i>	Electrocompetent <i>E. coli</i>
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO [®] vector	1 µl	1 µl
Final volume	6 µl	6 µl

*Store all reagents at -20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO[®] Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO[®] Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot[®] TOP10 Competent Cells**, next page.

Note: You may store the TOPO[®] Cloning reaction at -20°C overnight.

Transforming One Shot[®] TOP10 Competent Cells

Introduction

Once you have performed the TOPO[®] Cloning reaction, you will transform your pcDNA[™] Gateway[®] Directional TOPO[®] construct into competent *E. coli*. One Shot[®] TOP10 Chemically Competent *E. coli* (Box 2) are included with the 20 reaction kit to facilitate transformation, however, you may also transform electrocompetent cells (see page ix for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Blasticidin Selection

The presence of the EM7 promoter and the Blasticidin resistance gene in the pcDNA6.2/V5/GW/D-TOPO[®] vector allows for selection of *E. coli* transformants using Blasticidin. For selection, use Low Salt LB agar plates containing 100 µg/ml Blasticidin (see page 29 for a recipe). For Blasticidin to be active, the salt concentration of the medium must remain low (<90 mM) and the pH must be 7.0. Blasticidin is available separately from Invitrogen (see page ix for ordering information). Refer to the **Appendix**, page 31 for instructions on how to prepare and store Blasticidin.

Materials Needed

You should have the following materials on hand before beginning:

- 42°C water bath (or electroporator with cuvettes, optional)
 - LB plates containing 50-100 µg/ml ampicillin (two for each transformation)
 - 37°C shaking and non-shaking incubator
-



Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
 - Warm the vial of S.O.C. medium from Box 2 to room temperature.
 - Warm LB plates containing 50-100 µg/ml ampicillin at 37°C for 30 minutes.
 - Thaw **on ice** 1 vial of One Shot[®] TOP10 cells from Box 2 for each transformation.
-

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
 2. Incubate on ice for 5 to 30 minutes.
Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
 3. Heat-shock the cells for 30 seconds at 42°C without shaking.
 4. Immediately transfer the tubes to ice.
 5. Add 250 µl of room temperature S.O.C. medium.
 6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
 7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
 8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 15). Refer to the **Troubleshooting** section on page 23 if you have problems obtaining transformants.
-

Transformation by Electroporation

Use **ONLY** electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
 2. Electroporate your samples using your own protocol and your electroporator.
Note: If you have problems with arcing, see the next page.
 3. Immediately add 250 µl of room temperature S.O.C. medium.
 4. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the ampicillin resistance gene.
 5. Spread 20-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
 6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 15). Refer to the **Troubleshooting** section on page 23 if you have problems obtaining transformants.
-

continued on next page

Transforming One Shot[®] TOP10 Competent Cells, continued



To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 μl (0.1 cm cuvettes) or 100 to 200 μl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
 - Reduce the pulse length by reducing the load resistance to 100 ohms
 - Ethanol precipitate the TOPO[®] Cloning reaction and resuspend in water prior to electroporation
-

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml ampicillin.
 2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).
 3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.
-

Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation and in frame with the C-terminal V5 epitope tag. The T7 Promoter primer and the TK polyA Reverse primer are included in the kit to help you sequence your insert (see the diagram on page 8 for the location of the priming sites).



Important

If you download the sequence for pcDNA3.2/V5/GW/D-TOPO® or pcDNA6.2/V5/GW/D-TOPO® from our Web site, note that the overhang sequence (GTGG) will be shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the T7 Promoter primer or the TK polyA Reverse primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
 2. Pick 5 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above.
 3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
 4. Amplify for 20 to 30 cycles.
 5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
 6. Visualize by agarose gel electrophoresis.
-

continued on next page

Analyzing Transformants, continued



Important

If you have problems obtaining transformants, the correct insert, or inserts in the correct orientation, refer to the **Troubleshooting** section (see page 23). We also recommend that you perform the control reactions described on pages 25-26. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C .

1. Streak the original colony out for single colony on LB plates containing 50-100 $\mu\text{g/ml}$ ampicillin.
 2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 $\mu\text{g/ml}$ ampicillin.
 3. Grow until culture reaches stationary phase.
 4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
 5. Store at -80°C .
-

Transfecting Cells

Introduction

This section provides general information for transfecting your pcDNA™ Gateway® Directional TOPO® construct into the mammalian cell line of choice. We recommend that you include a positive control vector (pcDNA™3.2/V5/GW-CAT or pcDNA™6.2/V5/GW-CAT) and a mock transfection (negative control) in your experiments to evaluate your results.

Plasmid Preparation

Once you have generated your expression clone, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (*e.g.* HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent (Catalog no. 11668-027) available from Invitrogen. For more information about Lipofectamine™ 2000 and the other transfection reagents available from Invitrogen, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 35).

Positive Control

pcDNA™3.2/V5/GW-CAT or pcDNA™6.2/V5/GW-CAT is provided as a positive control vector for mammalian cell transfection and expression (see page 34 for a map) and may be used to optimize recombinant protein expression levels in your cell line. These vectors allow expression of a C-terminally tagged chloramphenicol acetyl transferase (CAT) fusion protein that may be detected by Western blot or functional assay.

To propagate and maintain the plasmid:

1. Use the stock solution to transform a *recA*, *endA* *E. coli* strain like TOP10, DH5 α , or equivalent.
 2. Select transformants on LB agar plates containing 50-100 μ g/ml ampicillin.
 3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.
-

Detecting Recombinant Fusion Proteins

Introduction

You may express your gene of interest in either transiently transfected cells or stable cell lines (see page 20 for guidelines to create stable cell lines). You may use a functional assay or a Western blot analysis to detect your recombinant protein (see below).

Preparing Cell Lysates

To detect your fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. A sample protocol is provided below. Other protocols are suitable. To lyse cells:

1. Wash cell monolayers ($\sim 5 \times 10^5$ to 1×10^6 cells) once with phosphate-buffered saline (see page ix for ordering information).
2. Scrape cells into 1 ml PBS and pellet the cells at $1500 \times g$ for 5 minutes.
3. Resuspend in 50 μ l Cell Lysis Buffer (see the **Appendix**, page 30 for a recipe). Other cell lysis buffers are suitable. Vortex.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.

Note: You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.

5. Centrifuge the cell lysate at $10,000 \times g$ for 10 minutes at $+4^\circ\text{C}$ to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration.

Note: Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.

6. Add SDS-PAGE sample buffer (see the **Appendix**, page 30 for a recipe) to a final concentration of 1X and boil the sample for 5 minutes.
 7. Load 20 μ g of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.
-

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE[®] and Novex[®] Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 35).

continued on next page

Detecting Recombinant Fusion Proteins, continued

Detecting Fusion Proteins

To detect expression of your recombinant fusion protein by Western blot analysis, you may use the Anti-V5 antibodies available from Invitrogen (see page x for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 35).



Note

Due to the presence of the *attB2* site, there will be additional amino acids between your gene of interest and the C-terminal V5 epitope tag (see page 8 for a diagram). The C-terminal peptide containing the *attB2* site and the V5 epitope will add approximately 4 kDa to your protein.

Assay for CAT Protein

If you use pcDNA™3.2/V5/GW-CAT or pcDNA™6.2/V5/GW-CAT as a positive control vector, you may assay for CAT expression using your method of choice. Note that CAT is fused to the C-terminal V5 epitope tag so you can use Western blot analysis and an Anti-V5 antibody to detect expression of CAT. CAT Antiserum is also available separately from Invitrogen (see page ix for ordering information). Other commercial kits are available for assaying CAT expression. The molecular weight of the CAT fusion protein is approximately 30 kDa.

Creating Stable Cell Lines

Introduction

The pcDNA3.2/V5/GW/D-TOPO[®] and pcDNA6.2/V5/GW/D-TOPO[®] vectors contain the neomycin and Blasticidin resistance genes, respectively, to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your construct into the mammalian cell line of choice and select for foci using Geneticin[®] (pcDNA3.2/V5/GW/D-TOPO[®] only) or Blasticidin (pcDNA6.2/V5/GW/D-TOPO[®] only). General information and guidelines are provided below.



To obtain stable transfectants, we recommend that you linearize your pcDNA3.2/V5/GW/D-TOPO[®] or pcDNA6.2/V5/GW/D-TOPO[®] construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts elements necessary for expression in mammalian cells. To linearize your construct, cut at a unique site that is not located within a critical element or within your gene of interest.

Geneticin[®]

Geneticin[®] blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial aminoglycoside phosphotransferase gene (APH), derived from Tn5, results in detoxification of Geneticin[®] (Southern and Berg, 1982). Geneticin[®] is available separately from Invitrogen (see page ix for ordering information).

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *bsd* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a nontoxic deaminohydroxy derivative (Izumi *et al.*, 1991). For more information about Blasticidin, see page 31. Blasticidin is available separately from Invitrogen (see page ix for ordering information).

continued on next page

Creating Stable Cell Lines, continued

Determination of Antibiotic Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of antibiotic (Geneticin[®] or Blastidicin) required to kill your untransfected host cell line. Test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Let cells attach overnight before adding selective medium.
2. For each antibiotic, prepare a set of 6-7 plates.
3. For Blastidicin selection, prepare a stock solution of 5-10 mg of Blastidicin in sterile water. Filter-sterilize the solution. For Geneticin[®] selection, prepare Geneticin[®] in a buffered solution (*e.g.* 100 mM HEPES, pH 7.3).
4. Add the following concentrations of antibiotic to each plate:
 - For Blastidicin selection, test 0, 1, 3, 5, 7.5, and 10 µg/ml Blastidicin
 - For Geneticin[®] selection, test 0, 50, 125, 250, 500, 750, and 1000 µg/ml Geneticin[®]
5. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
6. Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that prevents growth within 1-3 weeks after addition of the antibiotic.

Geneticin[®] Selection Guidelines

Once you have determined the appropriate Geneticin[®] concentration to use for selection, you can generate a stable cell line expressing your **pcDNA3.2/V5/GW/D-TOPO[®]** construct. Use as follows:

1. Prepare Geneticin[®] in a buffered solution (*e.g.* 100 mM HEPES, pH 7.3).
2. Use the predetermined concentration of Geneticin[®] in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Cells will divide once or twice in the presence of lethal doses of Geneticin[®], so the effects of the drug take several days to become apparent. Complete selection can take from 2 to 3 weeks of growth in selective medium.

Blasticidin Selection Guidelines

Once you have determined the appropriate Blasticidin concentration to use for selection, you can generate a stable cell line expressing your **pcDNA6.2/V5/GW/D-TOPO[®]** construct. Use as follows:

1. Prepare a stock solution of 5-10 mg/ml of Blasticidin in sterile water. Filter-sterilize the solution.
2. Use the predetermined concentration of Blasticidin in complete medium.
3. Cells differ in their susceptibility to Blasticidin. Complete selection can take as little as 10 days of growth in selective medium.

Refer to the **Appendix**, page 31 for instructions on how to prepare and store Blasticidin.

Generating an Entry Clone

Introduction

Once you have TOPO® Cloned your gene of interest into a pcDNA™ Gateway® Directional TOPO® vector, you may perform a BP recombination reaction between your expression construct and a Gateway® donor vector to generate an entry clone. Once you generate an entry clone, your gene of interest may then be easily shuttled into a large selection of destination vectors using the LR recombination reaction. Refer to the general guidelines below before using your pcDNA/V5/GW/D-TOPO® construct with the Gateway® Technology. Refer to the Gateway® Technology manual for detailed information on performing the BP recombination reaction, transforming competent cells, and selecting entry clones.

Recombining the Expression Clone with a Donor Vector

Before performing the BP reaction, consider the following points:

- The C-terminal V5 epitope tag will not be recombined into the entry clone. If you wish to fuse your gene of interest to an epitope tag, the tag will need to be provided by the destination vector in the LR recombination reaction.
 - If you cloned the gene of interest to be in frame with the C-terminal tag of one of the pcDNA/V5/GW/D-TOPO® vectors, the gene will remain in frame with any C-terminal tags provided by the destination vector following the LR recombination reaction. Note that the gene will not contain a stop codon in the entry clone. The stop codon will need to be provided by the destination vector in the LR recombination reaction.
-

Experimental Outline

To generate an entry clone, you will:

1. Perform a BP recombination reaction between your pcDNA/V5/GW/D-TOPO® expression clone and an *attP*-containing donor vector (see below)
 2. Transform the reaction mixture into a suitable *E. coli* host
 3. Select for entry clones
-

Gateway® Donor Vectors

Invitrogen offers a variety of Gateway® donor vectors to help you generate an entry clone containing your gene of interest (see page ix for ordering information). For more information about the vectors available, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 35).

Materials Needed

Be sure to have the following materials on hand before performing the BP recombination reaction. For more details, refer to the Gateway® Technology manual.

- pcDNA/V5/GW/D-TOPO® expression clone
 - pDONR™ vector (see page ix for ordering information)
 - BP Clonase™ enzyme mix (see page ix for ordering information)
 - 5X BP Clonase Reaction Buffer (supplied with the BP Clonase™ enzyme mix)
 - 2 µg/µl Proteinase K solution (supplied with the BP Clonase™ enzyme mix)
-

Troubleshooting

TOPO[®] Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO[®] Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions in parallel with your samples (see pages 25-26).

Problem	Reason	Solution
Few or no colonies obtained from sample reaction and the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO [®] vector used in the TOPO [®] Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO [®] vector.
	Too much PCR product used in the TOPO [®] Cloning reaction	<ul style="list-style-type: none"> Dilute the PCR product. Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO[®] vector.
	PCR product too dilute	<ul style="list-style-type: none"> Concentrate the PCR product. Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO[®] vector.
	PCR primers contain 5' phosphates	Do not add 5' phosphates to your PCR primers.
	Incorrect PCR primer design	<ul style="list-style-type: none"> Make sure that the forward PCR primer contains the sequence CACC at the 5' end. Make sure that the reverse PCR primer does not contain the sequence CACC at the 5' end.
	Used <i>Taq</i> polymerase or a <i>Taq</i> /proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Long PCR product	<ul style="list-style-type: none"> Increase the incubation time of the TOPO[®] reaction from 5 minutes to 30 minutes. Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (<i>i.e.</i> does not run as a single, discrete band on an agarose gel)	<ul style="list-style-type: none"> Optimize your PCR using the proofreading polymerase of choice. Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
Cloning large pool of PCR products or a toxic gene	<ul style="list-style-type: none"> Increase the incubation time of the TOPO[®] reaction from 5 minutes to 30 minutes. Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO[®] vector. 	

continued on next page

Troubleshooting, continued

TOPO[®] Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence CACC at the 5' end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5' end	Make sure that the reverse PCR primer does not contain the sequence CACC at the 5' end.
Large number of incorrect inserts cloned	PCR reaction contains artifacts (<i>i.e.</i> does not run as a single, discrete band on an agarose gel)	<ul style="list-style-type: none"> Optimize your PCR using the proofreading polymerase of choice. Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Incorrect PCR primer design	<ul style="list-style-type: none"> Make sure that the forward PCR primer contains the sequence CACC at the 5' end. Make sure that the reverse PCR primer does not contain the sequence CACC at the 5' end.
Few or no colonies obtained from sample reaction and the transformation control gave no colonies	One Shot [®] competent <i>E. coli</i> stored incorrectly	Store One Shot [®] competent <i>E. coli</i> at -80°C. If you are using another <i>E. coli</i> strain, follow the manufacturer's instructions.
	One Shot [®] transformation protocol not followed correctly	Follow the One Shot [®] transformation protocol provided on page 13.
	Insufficient amount of <i>E. coli</i> plated	Increase the amount of <i>E. coli</i> plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you TOPO® Clone to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50-100 µg/ml ampicillin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
<u>Thermostable polymerase (1-2.5 units/µl)</u>	<u>1 µl</u>
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible.
 5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.
-

continued on next page

Performing the Control Reactions, continued

Control TOPO[®] Cloning Reactions

Using the control PCR product produced on the previous page and a pcDNA[™] Gateway[®] Directional TOPO[®] vector, set up two 6 μ l TOPO[®] Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

1. Set up control TOPO[®] Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Water	4 μ l	3 μ l
Salt Solution	1 μ l	1 μ l
Control PCR Product	--	1 μ l
pcDNA/V5/GW/D-TOPO [®]	1 μ l	1 μ l
Final volume	6 μ l	6 μ l

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 μ l of each reaction into separate vials of One Shot[®] TOP10 cells (see page 13).
4. Spread 50-200 μ l of each transformation mix onto LB plates containing 50-100 μ g/ml ampicillin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pcDNA3.2/V5/GW/D-TOPO [®]	<i>Ava</i> I	Correct orientation: 4398, 1883 Reverse orientation: 4973, 1308 Empty vector: 5532
pcDNA6.2/V5/GW/D-TOPO [®]	<i>Ava</i> I	Correct orientation: 4028, 1883 Reverse orientation: 4603, 1308 Empty vector: 5162

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation. Relatively few colonies should be produced in the vector-only reaction.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot[®] TOP10 competent cells. Transform one vial of One Shot[®] TOP10 cells with 10 μ g of pUC19 using the protocol on page 13. Plate 10 μ l of the transformation mixture plus 20 μ l of S.O.C. on LB plates containing 100 μ g/ml ampicillin. Transformation efficiency should be $\sim 1 \times 10^9$ cfu/ μ g DNA.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below.



Note

The cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 9).

Using the S.N.A.P.[™] Gel Purification Kit

The S.N.A.P.[™] Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.
Note: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
 2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
 3. Add 1.5 volumes Binding Buffer.
 4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.[™] column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
 5. If you have solution remaining from Step 3, repeat Step 4.
 6. Add 900 µl of the Final Wash Buffer.
 7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
 8. Repeat Step 7.
 9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO[®] Cloning reaction and proceed as described on page 11.
-

Quick S.N.A.P.[™] Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.[™] column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO[®] Cloning reaction (page 11). Be sure to make the gel slice as small as possible for best results.

continued on next page

Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 11.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® TOP10 cells using the method on page 13.



Note

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Recipes

LB (Luria-Bertani) Medium and Plates

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (50-100 µg/ml ampicillin) if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
 2. Autoclave on liquid cycle for 20 minutes at 15 psi.
 3. After autoclaving, cool to ~55°C, add antibiotic (50-100 µg/ml of ampicillin), and pour into 10 cm plates.
 4. Let harden, then invert and store at +4°C.
-

Low Salt LB Medium with Blasticidin

Low Salt LB Medium:

10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.0 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
 2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
 3. Allow the medium to cool to at least 55°C before adding the Blasticidin to 100 µg/ml final concentration.
 4. Store plates at +4°C in the dark. Plates containing Blasticidin are stable for up to 2 weeks.
-

continued on next page

Recipes, continued

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions.
For 100 ml, combine

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml
2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

To prevent proteolysis, you may add 1 mM PMSF, 1 μ M leupeptin, or 0.1 μ M aprotinin before use.

4X SDS-PAGE Sample Buffer

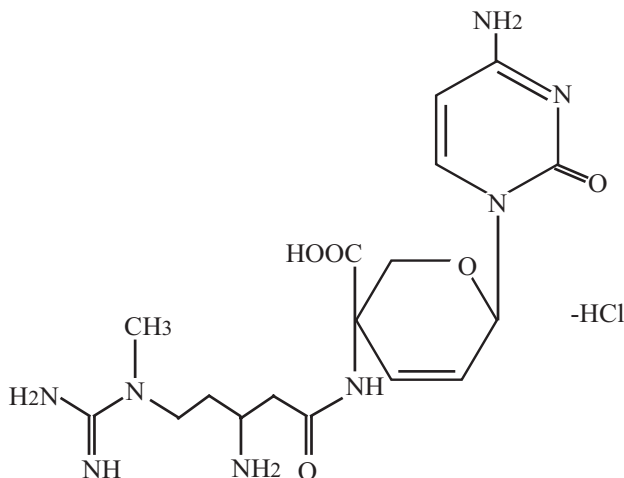
1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β -mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g
 2. Bring the volume to 10 ml with sterile water.
 3. Aliquot and freeze at -20°C until needed.
-

Blasticidin

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5 \cdot HCl$, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (e.g. a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

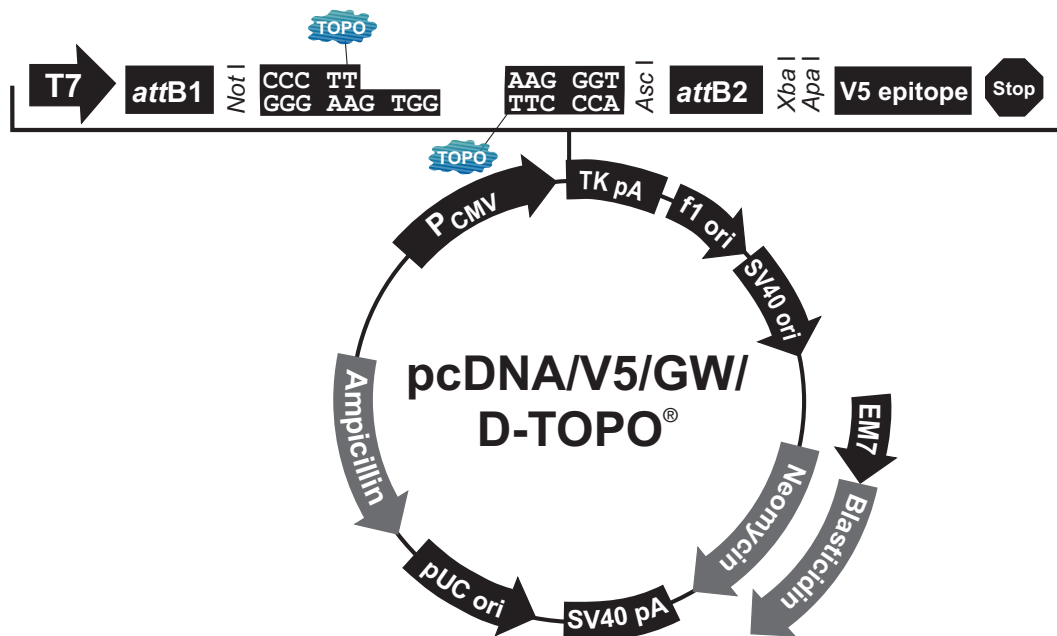
Blasticidin may be obtained separately from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/ml.

- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at -20°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1-2 weeks at +4°C and 6-8 weeks at -20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at +4°C for up to 2 weeks.
- Medium containing Blasticidin may be stored at +4°C for up to 2 weeks.

Map and Features of pcDNA/V5/GW/D-TOPO[®] Vectors

Map

The map below shows the elements of the pcDNA/V5/GW/D-TOPO[®] vectors. The complete sequences of these vectors are available for downloading from www.invitrogen.com or by contacting Technical Service (page 35).



Comments for:	pcDNA3.2/V5/GW/D-TOPO [®] 5532 nucleotides	pcDNA6.2/V5/GW/D-TOPO [®] 5162 nucleotides
CMV promoter:	232-819	232-819
T7 promoter/priming site:	863-882	863-882
attB1 site:	911-935	911-935
TOPO [®] recognition site 1:	947-951	947-951
Overhang sequence (c):	952-955	952-955
TOPO [®] recognition site 2:	956-960	956-960
attB 2 site:	972-996	972-996
V5 epitope:	1022-1063	1022-1063
TK polyadenylation signal:	1090-1361	1090-1361
TK polyA reverse priming site:	1097-1115	1097-1115
f1 origin:	1397-1825	1397-1825
SV40 early promoter and origin:	1852-2160	1852-2160
Neomycin resistance gene:	2235-3029	---
EM7 promoter:	---	2215-2281
Blasticidin resistance gene:	---	2282-2680
SV40 early polyadenylation signal:	3205-3335	2838-2968
pUC origin (c):	3718-4391	3351-4021
Ampicillin (<i>b/a</i>) resistance gene (c):	4536-5396	4166-5026
<i>bla</i> promoter (c):	5397-5495	5027-5125

(c) = complementary strand

continued on next page

Map and Features of pcDNA/V5/GW/D-TOPO[®] Vectors, continued

Features

pcDNA3.2/V5/GW/D-TOPO[®] (5532 bp) and pcDNA6.2/V5/GW/D-TOPO[®] (5162 bp) contain the following elements. Features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson <i>et al.</i> , 1989; Boshart <i>et al.</i> , 1985; Nelson <i>et al.</i> , 1987)
T7 promoter/priming site	Allows <i>in vitro</i> transcription in the sense orientation and sequencing through the insert
<i>attB1</i> and <i>attB2</i> sites	Allows recombinational cloning of the gene of interest to generate an entry clone
TOPO [®] Cloning site (directional)	Allows directional cloning of your PCR product in frame with C-terminal V5 epitope tag, if desired
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 antibodies (Southern <i>et al.</i> , 1991)
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985)
TK polyA reverse priming site	Allows sequencing of the insert
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin or Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene (pcDNA3.2/V5/GW/D-TOPO [®] only)	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
EM7 promoter (pcDNA6.2/V5/GW/D-TOPO [®] only)	Allows expression of the Blasticidin resistance gene in <i>E. coli</i>
Blasticidin (<i>bsd</i>) resistance gene (pcDNA6.2/V5/GW/D-TOPO [®] only)	Allows selection of stable transfectants in mammalian cells (Kimura <i>et al.</i> , 1994)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in <i>E. coli</i>
Ampicillin (<i>bla</i>) resistance gene (β-lactamase)	Allows selection of transformants in <i>E. coli</i>

Map of pcDNA™ 3.2/V5/GW-CAT and pcDNA™ 6.2/V5/GW-CAT

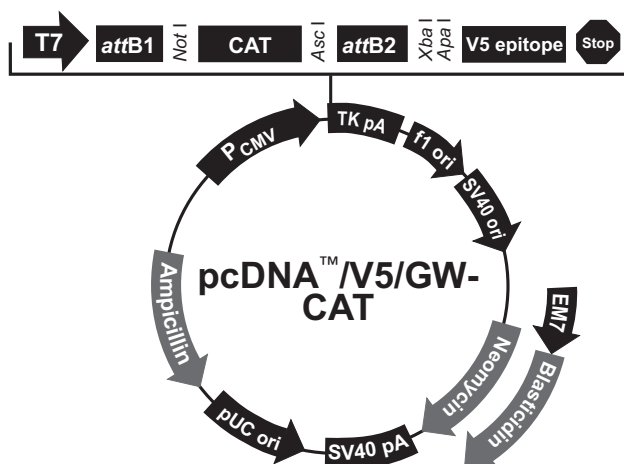
Description

pcDNA™3.2/V5/GW-CAT (6188 bp) and pcDNA™6.2/V5/GW-CAT (5818 bp) are control vectors expressing chloramphenicol acetyltransferase (CAT). Each vector was constructed using the Gateway® LR recombination reaction between an entry clone containing the CAT gene and the respective destination vector. CAT is expressed as a fusion to the V5 epitope tag. The molecular weight of the CAT fusion protein is approximately 30 kDa.

For more information on the Gateway® Technology, refer to the Gateway® Technology manual. For more information on pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST, refer to the pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST Gateway® Vectors manual.

Map

The map below shows the elements of pcDNA™3.2/V5/GW-CAT and pcDNA™6.2/V5/GW-CAT. **The complete sequences of these vectors are available for downloading from www.invitrogen.com or by contacting Technical Service (page 35).**



Comments for:	pcDNA™ 3.2/V5/GW-CAT 6188 nucleotides	pcDNA™ 6.2/V5/GW-CAT 5818 nucleotides
CMV promoter:	232-819	232-819
T7 promoter/priming site:	863-882	863-882
attB1 site:	911-935	911-935
CAT ORF:	955-1611	955-1611
attB2 site:	1628-1652	1628-1652
V5 epitope:	1678-1719	1678-1719
TK polyadenylation signal:	1746-2017	1746-2017
TK polyA reverse priming site:	1753-1771	1753-1771
f1 origin:	2053-2481	2053-2481
SV40 early promoter and origin:	2508-2816	2508-2816
Neomycin resistance gene:	2891-3685	---
EM7 promoter:	---	2871-2937
Blasticidin resistance gene:	---	2938-3336
SV40 early polyadenylation signal:	3861-3991	3494-3624
pUC origin (c):	4374-5047	4007-4677
Ampicillin (<i>bla</i>) resistance gene (c):	5192-6052	4822-5682
<i>bla</i> promoter (c):	6053-6151	5683-5781

(c) = complementary strand

Technical Service

Web Resources



Visit the Invitrogen website at www.invitrogen.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
 - Complete technical service contact information
 - Access to the Invitrogen Online Catalog
 - Additional product information and special offers
-

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

Corporate Headquarters:

Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_support@invitrogen.com

Japanese Headquarters:

Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:

Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

MSDS

MSDSs (Material Safety Data Sheets) are available on our website at www.invitrogen.com/msds.

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Introduction

Use of the pcDNA™ Gateway® Directional TOPO® Expression Kits is covered under the licenses detailed below.

Limited Use Label License No. 5: Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For products that are subject to multiple limited use label licenses, the terms of the most restrictive limited use label license shall control. Life Technologies Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Life Technologies Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information about purchasing a license to use this product or the technology embedded in it for any use other than for research use please contact Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008; Phone (760) 603-7200 or e-mail: outlicensing@lifetech.com.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 19: Gateway® Cloning Products

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any method claims in the foregoing patents or patent applications, or to use this product with any recombination sites other than those purchased from Life Technologies Corporation or its authorized distributor. The right to use methods claimed in the foregoing patents or patent applications with this product for research purposes only can only be acquired by the use of Clonase™ purchased from Life Technologies Corporation or its authorized distributors. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Notwithstanding the preceding, any buyer who is employed in an academic or government institution may transfer materials made with this product to a third party who has a license from Life Technologies under the patents identified above to distribute such materials. Transfer of such materials and/or information to collaborators does not convey rights to practice any methods claimed in the foregoing patents or patent applications. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of (i) this product, (ii) any of its components, or (iii) a method claim of the foregoing patents, was used in the manufacture of such product. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the use of this product to manufacture a protein for sale, provided that no method claim in the above patents was used in the manufacture of such protein. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200.

Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 39.

continued on next page

Purchaser Notification, continued

**Limited Use Label
License No. 51:
Blasticidin and the
Blasticidin
Selection Marker**

Blasticidin and the blasticidin resistance gene (bsd) are the subject of U.S. Patent No. 5,527,701 sold under patent license for research purposes only. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

continued on next page

Gateway[®] Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway[®] Technology.

Gateway[®] Entry Clones

Invitrogen understands that Gateway[®] entry clones, containing *attL1* and *attL2* sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway[®] Expression Clones

Invitrogen also understands that Gateway[®] expression clones, containing *attB1* and *attB2* sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway[®] expression clones for a nominal fee (\$10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway[®] entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway[®] Technology, and that the purchase of Gateway[®] Clonase[™] from Invitrogen is required for carrying out the Gateway[®] recombinational cloning reaction. This should allow researchers to readily identify Gateway[®] containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway[®] Technology, including Gateway[®] clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pcDNA™ Gateway® Directional TOPO® Expression Kits.

Vectors

pcDNA™3.2/V5/GW (parental vector of pcDNA3.2/V5/GW/D-TOPO®), pcDNA™6.2/V5/GW (parental vector of pcDNA6.2/V5/GW/D-TOPO®), and their respective expression control plasmids are qualified by restriction digest prior to adaptation with topoisomerase I. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

TOPO® Cloning Efficiency

The pcDNA™ Gateway® Directional TOPO® vectors are lot-qualified using the control reagents included in the kit. Under conditions described on pages 25-26, a 750 bp control PCR product is amplified using a forward primer containing CACC at its 5' end and a reverse primer. The PCR product is TOPO® Cloned into the pcDNA3.2/V5/GW/D-TOPO® or pcDNA6.2/V5/GW/D-TOPO® vector and transformed into the One Shot® TOP10 chemically competent *E. coli* included with the kit.

Each lot of vector should yield greater than 90% cloning efficiency. Forty transformants are characterized using restriction digest. Of the transformants characterized, greater than 90% should contain an insert in the correct orientation. Transformants are further qualified in a recombination assay using Gateway® BP Clonase™ enzyme mix.

Primers

Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® TOP10 Chemically Competent *E. coli*

1. One Shot® TOP10 chemically competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1×10^9 cfu/µg plasmid DNA.
 2. To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
 3. Untransformed cells are plated on LB plates containing 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.
-

References

- Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989). Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. *J. Biol. Chem.* *264*, 8222-8229.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). *Current Protocols in Molecular Biology* (New York: Greene Publishing Associates and Wiley-Interscience).
- Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. *Cell* *41*, 521-530.
- Capone, J. P., Sharp, P. A., and RajBhandary, U. L. (1985). Amber, Ochre and Opal Suppressor tRNA Genes Derived from a Human Serine tRNA Gene. *EMBO J.* *4*, 213-221.
- Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. *Mol. Cell. Biol.* *7*, 2745-2752.
- Cheng, C., and Shuman, S. (2000). Recombinogenic Flap Ligation Pathway for Intrinsic Repair of Topoisomerase IB-Induced Double-Strand Breaks. *Mol. Cell. Biol.* *20*, 8059-8068.
- Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. *Nucleic Acids Res.* *15*, 1311-1326.
- Cole, C. N., and Stacy, T. P. (1985). Identification of Sequences in the Herpes Simplex Virus Thymidine Kinase Gene Required for Efficient Processing and Polyadenylation. *Mol. Cell. Biol.* *5*, 2104-2113.
- Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. *Proc. West. Pharmacol. Soc.* *32*, 115-121.
- Felgner, P. L. a., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. *Nature* *337*, 387-388.
- Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991). Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. *Exp. Cell Res.* *197*, 229-233.
- Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994). Blasticidin S Deaminase Gene from *Aspergillus terreus* (*BSD*): A New Drug Resistance Gene for Transfection of Mammalian Cells. *Biochim. Biophys. ACTA* *1219*, 653-659.
- Kozak, M. (1987). An Analysis of 5'-Noncoding Sequences from 699 Vertebrate Messenger RNAs. *Nucleic Acids Res.* *15*, 8125-8148.
- Kozak, M. (1991). An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. *J. Cell Biology* *115*, 887-903.
- Kozak, M. (1990). Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. *Proc. Natl. Acad. Sci. USA* *87*, 8301-8305.

continued on next page

References, continued

- Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. *Annu. Rev. Biochem.* 58, 913-949.
- Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987). Negative and Positive Regulation by a Short Segment in the 5'-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. *Molec. Cell. Biol.* 7, 4125-4129.
- Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. *BioTechniques* 6, 742-751.
- Shuman, S. (1994). Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using Vaccinia DNA Topoisomerase. *J. Biol. Chem.* 269, 32678-32684.
- Shuman, S. (1991). Recombination Mediated by Vaccinia Virus DNA Topoisomerase I in *Escherichia coli* is Sequence Specific. *Proc. Natl. Acad. Sci. USA* 88, 10104-10108.
- Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. *J. Gen. Virol.* 72, 1551-1557.
- Southern, P. J., and Berg, P. (1982). Transformation of Mammalian Cells to Antibiotic Resistance with a Bacterial Gene Under Control of the SV40 Early Region Promoter. *J. Molec. Appl. Gen.* 1, 327-339.
- Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958). Blasticidin S, A New Antibiotic. *The Journal of Antibiotics, Series A* 11, 1-5.
- Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. *Cell* 11, 223-232.
- Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965). Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. *J. Biochem (Tokyo)* 57, 667-677.
-

©2002-2006, 2010 Invitrogen Corporation. All rights reserved.

For research use only. Not intended for any animal or human therapeutic or diagnostic use.



Corporate Headquarters

Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech.service@invitrogen.com

For country-specific contact information visit our web site at www.invitrogen.com